Specific leukotriene formation by purified human eosinophils and neutrophils

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Received 30 December 1983

Human granulocytes isolated from peripheral blood have been described to synthesize both LTB_4 and LTC_4 from arachidonic acid. We have observed that the amount of LTC_4 produced by human granulocyte preparations is strongly dependent on the relative amount of eosinophils. To investigate a possibly significant difference in leukotriene synthesis of the eosinophilic and neutrophilic granulocytes, we developed a purification method to isolate both cell types from granulocytes obtained from the blood of healthy donors. Leukotrienes were generated by incubation of the purified cells with arachidonic acid, calcium ionophore A23187, calcium-chloride and reduced glutathione. Surprisingly, eosinophils were found to produce almost exclusively the spasmogenic LTC_4. In contrast, neutrophils produce almost exclusively the chemotactic LTB_4, its ω-hydroxylated metabolite 20-hydroxy-LTB_4 and two non-enzymically formed LTB_4 isomers.

Leukotriene *Granulocyte* *Eosinophil* *Neutrophil* *Lipoxygenase* *Asthma*

1. INTRODUCTION

Leukotrienes constitute an important class of biologically active arachidonic acid metabolites, formed via the lipoxygenase pathway. Several types of human blood and tissue cell e.g., granulocytes [1,2], lymphocytes [3], macrophages [4,5] and mast cells [6], are capable of synthesizing leukotrienes from arachidonic acid. Leukotriene C_4 (LTC_4: 5(*S*)-hydroxy-6(*R*)-*S*-glutathionyl-7,9*trans*-11,14-*cis*-eicosatetraenoic acid) and leukotriene D_4 (LTD_4: 5(*S*)-hydroxy-6(*R*)-*S*-cysteinylglycyl-7,9-*trans*-11,14-*cis*-eicosatetraenoic acid) have been shown to be strongly bronchoconstrictive in vitro [7] as well as in vivo [8] and therefore may play an important role as mediators in (immediate) hypersensitivity reactions. Leukotriene B_4 (LTB_4: 5(*S*), 12(*R*)-dihydroxy-6,14-*cis*-8,10-*trans*eicosatetraenoic acid) possesses a pronounced chemotactic activity for human neutrophils [9] and eosinophils [10]. Since eosinophils probably play an important role in hypersensitivity reactions, it is of interest to know whether these cells are capable of producing leukotrienes and if so whether the leukotriene formation by eosinophils differs qualitatively and/or quantitatively from the formation of leukotrienes by neutrophils.

Recently the production of leukotrienes by purified horse eosinophils [11], murine eosinophils [12] and human eosinophils [13,14] has been described. Authors in [12,13] observed that murine and human eosinophils produce leukotrienes of the 5- and 15-series in almost equal amounts. Authors in [11] reported that horse eosinophils produce

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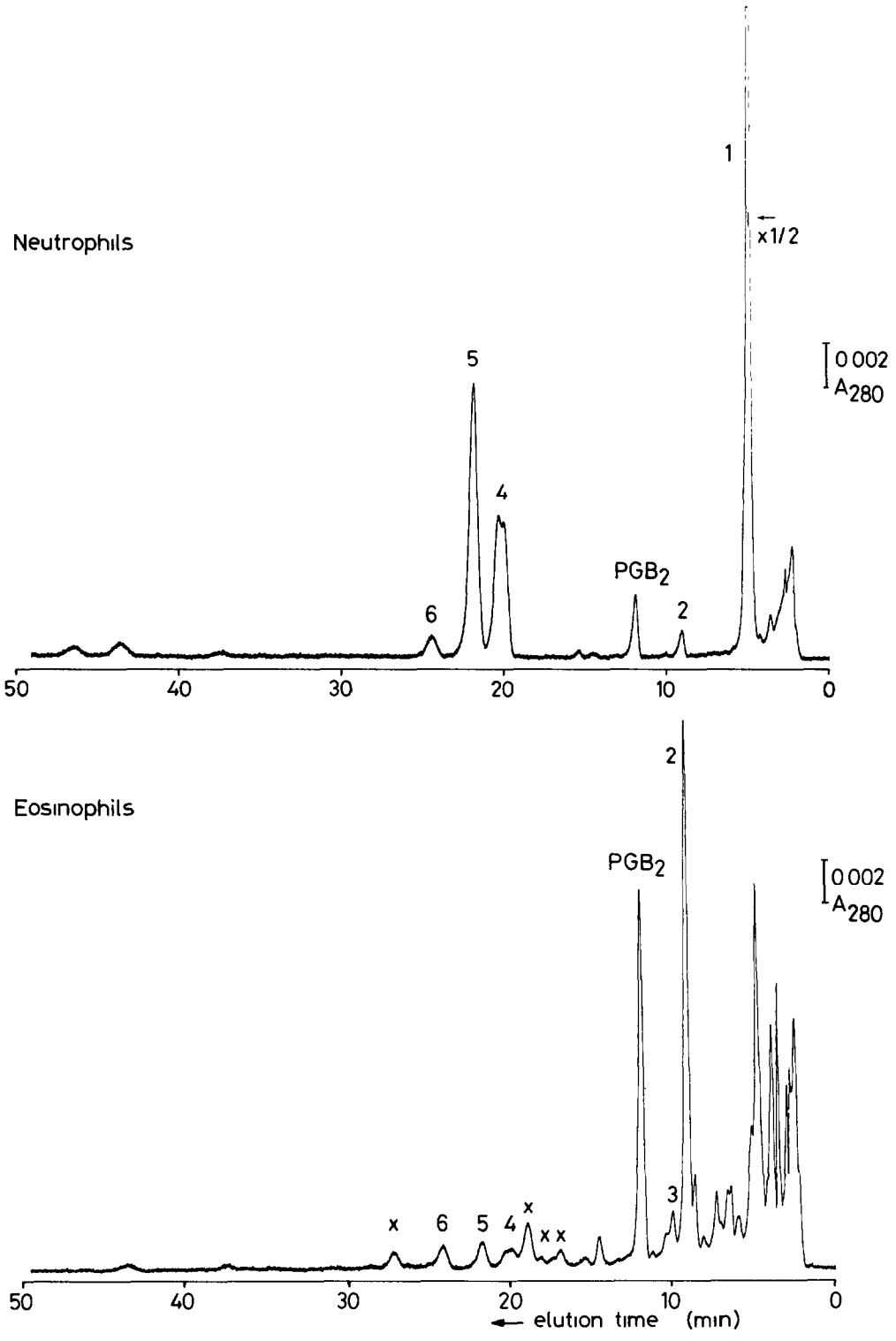
00145793/84/$3 00


Neutrophils
1
x1/2
5
0.002
A280
4
PGB2
6
2
50
40
30
20
10
0
Eosinophils
2
PGB2
0.002
A280
3
x
6
5
4
x
x x
50
40
30
20
10
0
elution time (min)

LTB_4 and LTB_4 isomers as well as about 4-times as much sulfidopeptide leukotrienes per cell as horse neutrophils. Authors in [14] found that – in the absence of added glutathione – human eosinophils produce small amounts of LTB_4, LTC_4 and LTD_4.

So far, leukotriene formation by human eosinophils and neutrophils under optimal conditions has not been compared in detail.

Here, a method has been developed to purify human eosinophils and neutrophils from granulocyte preparations obtained from quantities as small as 100 ml blood from healthy donors. The formation of leukotrienes by eosinophils and neutrophils has been compared in the presence of sufficient amounts of glutathione to enable optimal production of sulfidopeptide leukotrienes.

2. MATERIALS AND METHODS

2.1. *Materials*

Arachidonic acid (purity >99%) was purchased from Fluka (Buchs, Switzerland) and Nu Chek Prep, Inc. (MN). Calcium ionophore A23187, reduced glutathione, PGB_2 and 4-hydroxy-2,2,6,6-tetramethylpiperidinooxy free radical were from Sigma (St. Louis, MO). Percoll (1.129 g/ml) and Ficoll-Paque (1.077 g/ml) were obtained from Pharmacia (Uppsala). Solvents, which were all of HPLC quality, and octadecyl reversed-phase extraction columns (6 ml) were purchased from Baker (Deventer, The Netherlands). Synthetic LTB_4, LTC_4 and LTD_4 were a kind gift of Dr J. Rokach (Merck-Frosst Laboratories, Pointe Claire/Dorval, Quebec). Human blood was obtained from healthy volunteers of the Red Cross Bloodbank Foundation (Utrecht).

2.2. *Purification of granulocytes*

Granulocytes were isolated as follows: platelet-rich plasma was removed after centrifugation of citrated blood at 275 × *g* for 15 min. After collection of the buffy coat, granulocytes were separated from lymphocytes and monocytes by centrifugation on Ficoll-Paque at 650 × *g* for 20 min. The remaining erythrocytes were removed by ammonium-chloride lysis at 0°C and subsequent centrifugation.

The purity of the cell preparations was found to be better than 97%. The content of eosinophils in these preparations ranged from 2 to 8%.

2.3. *Purification of eosinophils and neutrophils*

Eosinophils and neutrophils were separated from each other by two subsequent centrifugations of a granulocyte preparation in isotonic Percoll solutions with densities of 1.085 and 1.088 g/ml at 1000 × *g* for 10 min at room temperature. 96 ± 3% pure neutrophils appeared at the top of the 1.085 g/ml Percoll solution, while the eosinophils and some neutrophils were present as a small pellet on the bottom.

After resuspension of the eosinophil pellet in a 1.088 g/ml Percoll solution and centrifugation at 1000 × *g* for 10 min at room temperature, 96 ± 4% pure eosinophils appeared on the bottom of the tube.

Recoveries were 37 ± 16% and 85 ± 10% for eosinophils and neutrophils, respectively ($n = 5$). Cell integrity was measured with the vital stains fluorescein diacetate and ethidium bromide [15,16]. Further support for cell integrity was obtained by the absence of lactate dehydrogenase and β-glucuronidase activities in the supernatants of isolated, unstimulated cells.

2.4. *Incubation procedure*

Eosinophils or neutrophils were suspended in Dulbecco's salt solution (pH 7.4) at a concentration of approx. 10^7 cells/ml, preincubated at 37°C for 5 min and then incubated at 37°C for 5 min with 80 μM arachidonic acid, 20 μM calcium ionophore A23187, 1 mM (extra) $CaCl_2$ and 5 mM

←

Fig. 1. RP-HPLC chromatograms of the leukotrienes produced from arachidonic acid (80 μM) by 8×10^6 neutrophils (purity 99%) or eosinophils (purity 98%) isolated from the same granulocyte preparation. Solvent system: tetrahydrofuran–methanol–water–acetic acid (25:30:45:0.1, by vol.), pH 5.5, flow rate: 0.9 ml/min. UV spectra were recorded of all peaks. Synthetic LTB_4 abd LTC_4 were used as references. Identification of peaks: 1, 20-hydroxy-LTB_4; 2, LTC_4; 3, 11*t*-LTC_4; 4, 12*epi*,6*t*-LTB_4 + 6*t*-LTB_4; 5, LTB_4; 6, 12*epi*,6*t*,8*c*-LTB_4 (double-dioxygenation product). X, 15-series leukotrienes. NB, the peak in the eosinophil chromatogram having a position almost identical with the 20-hydroxy-LTB_4 peak in the neutrophil chromatogram does not possess a characteristic triene UV spectrum.

reduced glutathione. Reactions were stopped by the addition of ice to the incubation mixture.

2.5. *Sample preparation*

The incubation mixture was centrifuged at 46 000 × *g* for 10 min at 4°C. For the extraction of the formed leukotrienes octadecyl (C18) reversed-phase extraction columns (6 ml) were used, which were first washed with methanol and water, respectively, according to the manufacturers instructions. After applying the cell supernatant (pH 7.4) to the column and washing with water, the absorbed leukotrienes were eluted with methanol (usually 3 ml). Recovery for all leukotrienes was found to be better than 85%.

After addition of the radical scavenger 4-hydroxy-2,2,6,6-tetramethylpiperidinooxy free radical (2 μg/3 ml), the eluates were stored under nitrogen at −70°C until they were analyzed by RP-HPLC.

2.6. *Reversed-phase high-performance liquid chromatography (RP-HPLC)*

Leukotrienes were separated and quantified, as in [17] by isocratic RP-HPLC, using a Nucleosil 5C18 column (250 × 4.6 mm, Chrompack, Middelburg, The Netherlands) attached to a Perkin-Elmer Series 1 pump and a Perkin-Elmer LC85 detector. The solvent system was tetrahydrofuran–methanol–water–acetic acid (25:30:45:0.1, by vol.) which had been brought to pH 5.5 with ammonium hydroxide. The aqueous phase contained 0.1% EDTA to prevent binding of cations to the column. Such binding could lead to excessive retention of sulfidopeptide leukotrienes [18]. A flow rate of 0.9 ml/min was maintained and the effluent was monitored at 280 nm (leukotrienes) or 237 nm (monohydroxy acids). Peak areas were determined with a Hewlett-Packard 3390 A integrator and related to the internal standard PGB_2, using molar absorption coefficients ($M^{-1} \cdot cm^{-1}$) of 28 650 for PGB_2 and 40 000 for the leukotrienes at 280 nm and 29 500 for the monohydroxy acids at 237 nm [19].

3. RESULTS

Eosinophils and neutrophils were purified from

Table 1

Leukotriene formation from arachidonic acid by purified human eosinophils and neutrophils: Comparison of data of this study with those from the literature

	LTC_4	LTD_4	LTB_4	20-hydroxy-LTB_4	12*epi*,6*t*-LTB_4 + 6*t*-LTB_4	12*epi*,6*t*, 8*c*-LTB_4	15-series leukotrienes	LTC_4 + LTD_4 / LTB_4 + isomers	15-series / 5-series
Human neutrophils (here)[a]	3 ± 3	–	29 ± 5	45 ± 8	24 ± 6	3 ± 1	–	0.03	0.00
Human eosinophils (here)[a]	35 ± 13	–	4 ± 2	–	4 ± 2	2 ± 1	5 ± 5	3.50	0.11
Eosinophils (literature)[b]									
Human [14][c]	1.1	0.7	2.8	n.d.	10.0	n.d.	n.d.	0.14	n.d.
Human [13][d]	n.d.	n.d.	1.8	n.d.	6.9	5.6	11.8	n.d.	0.82
Murine [12][e]	n.d.	n.d.	1.1	n.d.	5.9	7.7	11 2	n d.	0.76
Horse [11][f]	11.1	1.1	6.5	n.d.	8.9	n.d.	n.d.	0.73	n.d.

[a] From 5 different granulocyte preparations, eosinophils and neutrophils have been purified and stimulated; mean values (expressed as 10^6 molecules released/cell) are given ± SD

[b] The literature data were originally expressed in ng leukotriene formed per 10^8 cells and were recalculated to 10^6 molecules of leukotriene formed per cell (no corrections have been made for losses during isolation)
Incubation periods: [d,e] and here: 5 min; [f] 15 min; [c] 20 min. [c,f] Glutathione has not been added; [d,e] LTC_4 was not taken into consideration

granulocytes obtained from 0.1 to 1.0 l human blood. Fig.1 shows RP-HPLC chromatograms of the leukotrienes formed by eosinophils or neutrophils purified from the same granulocyte preparation. The absolute quantity of each leukotriene produced per cell in 5 min is given in table 1. These data prove that almost all LTC_4 produced in vitro by human granulocytes is synthesized by the eosinophil population which accounts only for less than 5% of the granulocytes in healthy donors. On the other hand, the syntheses of LTB_4, 20-hydroxy-LTB_4 and the two non-enzymically formed LTB_4 isomers are almost exclusively due to the neutrophils. The relative amount of 20-hydroxy-LTB_4 was found to dependent on the length of the incubation period. After 20 min nearly all LTB_4 had been converted into 20-hydroxy-LTB_4. It is interesting to note that even after prolonged incubation of eosinophils 20-hydroxy-LTB_4 could not be detected.

Both neutrophils and eosinophils produce only very small amounts of the double-dioxygenation product 12*epi*,6*t*,8*c*-LTB_4 (sometimes referred to as 5S,12S-DHETE). Furthermore, eosinophils were found to produce minor quantities of leukotrienes formed from 15(*S*)-hydroperoxy-5,8,11-*cis*-13-*trans*-eicosatetraenoic acid (15HPETE), which were not formed by neutrophils under these conditions. The small amounts of the latter compounds did not allow structural characterization. However, it seems reasonable to assume that these leukotrienes are formed from 15HPETE, because the RP-HPLC chromatogram of the leukotrienes produced from 15HPETE by porcine lymphocytes shows peaks with identical retention times and UV spectra (unpublished). The major monohydroxy-eicosatetraenoic acid formed by eosinophils was 15-hydroxy-5,8,11-*cis*-13-*trans*-eicosatetraenoic acid (15HETE, elution time RP-HPLC: 67 min) whereas neutrophils were found to synthesize mainly 5-hydroxy-6-*trans*-8,11,14-*cis*-eicosatetraenoic acid (5HETE, elution time RP-HPLC: 92 min).

As 5-lipoxygenases have until now always been found to be Ca^{2+}-dependent [1,20] whereas 15-lipoxygenases have not [21,22], some incubations with eosinophils and neutrophils were done in the absence of calcium ionophore and of extracellular Ca^{2+}. Under these conditions the syntheses of LTC_4, LTB_4 and 5HETE were found to be strongly reduced, whereas the production of 15HETE and 15-series leukotrienes by eosinophils was not affected significantly.

Therefore, the effect of Ca^{2+} supplies additional evidence that eosinophilic LTC_4 is produced via the 5-lipoxygenase pathway. Without added glutathione the production of LTC_4 by eosinophils was reduced to approx. 20% of the amount formed in the presence of 5 mM glutathione. Therefore, it must be concluded that the eosinophil shows its potent and specific capacity to produce LTC_4 in vitro only when Ca^{2+}, calcium ionophore as well as glutathione are present in sufficient quantities.

4. DISCUSSION

This study demonstrates that human eosinophilic and neutrophilic granulocytes synthesize different types of leukotrienes. Until now, such a specificity for human eosinophils and neutrophils has not been reported. It is interesting to compare our results with literature data as compiled in table 1.

From these data it is obvious that the relative amounts of LTB_4, its isomers and 15-series leukotrienes formed by eosinophils have been overestimated because the production of LTC_4 has not been sufficiently stimulated [14] or LTC_4 formation has not been taken into consideration at all [12,13]. Therefore, these investigations conceal the specific potency of the eosinophil to produce large amounts of LTC_4.

Our finding that the eosinophilic granulocyte population is almost entirely responsible for the production of the spasmogenic LTC_4 by granulocytes, may throw new light on the role of the eosinophil in (immediate) hypersensitivity reactions. Frequently, asthmatic patients have high numbers of eosinophils in their lung tissue which is often reflected in an increased number of blood eosinophils. Until now, the role of the eosinophil in this disease was often regarded to be beneficial, because eosinophils have been shown to contain enzymes capable of inactivating LTC_4 [23,24] and histamine [25]. However, the here demonstrated potency of the eosinophil to produce large amounts of LTC_4 shows that – under certain conditions – this activity may prevail over a possibly beneficial activity.

ACKNOWLEDGEMENTS

We thank Dr D. Roos and Dr Pauline Walstra for helpful comments on the manuscript. This work was supported in part by grant 82.18 from the Netherlands Asthma Foundation.

REFERENCES

[1] Borgeat, P. and Samuelsson, B. (1979) Proc. Natl. Acad. Sci. USA 76, 2148–2152.
[2] Hansson, G. and Radmark, O. (1980) FEBS Lett. 122, 87–90.
[3] Goetzl, E.J. (1981) Biochem. Biophys. Res. Commun. 101, 344–350.
[4] Fels, A.O.S., Pawlowski, N.A., Cramer, E.B., King, T.K.C., Cohn, Z.A. and Scott, W.A. (1982) Proc. Natl. Acad. Sci. USA 79, 7866–7870.
[5] Damon, M., Chavis, C., Godard, P., Michel, F.B. and Crastes de Paulet, A. (1983) Biochem. Biophys. Res. Commun. 111, 518–524.
[6] MacGlashan, D.W., jr, Schleimer, R.P., Peters, S.P., Schulman, E.S., Adams III, G.K. and Newball, H.H. (1982) J. Clin. Invest. 70, 747–751.
[7] Dahlén, S.E., Hedqvist, P., Hammarstrom, S. and Samuelsson, B. (1980) Nature 288, 484–486.
[8] Holroyde, M.C., Altounyan, R.E.C., Cole, M., Dixon, M. and Elliot, E.V. (1981) Lancet 2 (8236), 17–18.
[9] Ford-Hutchinson, A.W., Bray, M.A., Doig, M.V., Shipley, M.E. and Smith, M.J.H. (1980) Nature 286, 264–265.
[10] Nagy, L., Lee, T.H., Goetzl, E.J., Pickett, W.C. and Kay, A.B. (1982) Clin. Exp. Immunol. 47, 541–547.
[11] Jorg, A., Henderson, W.R., Murphy, R.C. and Klebanoff, S.J. (1982) J. Exp. Med. 155, 390–402.
[12] Turk, J., Rand, T.H., Maas, R.L., Lawson, J.A., Brash, A.R., Roberts II, L.J., Colley, D.G. and Oates, J.A. (1983) Biochim. Biophys Acta 750, 78–90.
[13] Turk, J., Maas, R.L., Brash, A.R., Roberts II, L.J. and Oates, J.A. (1982) J. Biol. Chem. 257, 7068–7076.
[14] Henderson, W.R., Harley, J.B., Fauci, A.S. and Klebanoff, S.J. (1983) J. Allergy Clin. Immunol. 71, 138.
[15] Edidin, M. (1970) J. Immunol. 104, 1303–1306.
[16] Takasugi, M. (1971) Transplantation 12, 148–151.
[17] Verhagen, J., Walstra, P., Veldink, G.A., Vliegenthart, J.F.G. and Bruynzeel, P.L.B. (1984) Prostagl. Leukotr. Med., in press.
[18] Metz, S.A., Hall, M.E., Harper, T.W. and Murphy, R.C. (1982) J. Chromatogr. 233, 193–201.
[19] Van Os, C.P.A., Rijke-Schilder, G.P.M., Van Halbeek, H., Verhagen, J. and Vliegenthart, J.F.G. (1981) Biochim. Biophys. Acta 663, 177–193.
[20] Jakschik, B.A., Sun, F.F., Lee, L.-H. and Steinhoff, M.M. (1980) Biochem. Biophys. Res. Commun. 95, 103–110.
[21] Narumiya, S., Salmon, J.A., Cottee, F.H., Weatherley, B.C. and Flower, R.J. (1981) J. Biol. Chem. 256, 9583–9592.
[22] Schewe, T., Halangk, W., Hiebsch, C. and Rapoport, S.M. (1975) FEBS Lett. 60, 149–152.
[23] Wasserman, S.I , Goetzl, E.J. and Austen, F.K. (1975) J. Immunol. 114, 645–649.
[24] Goetzl, E.J. (1982) Biochem. Biophys. Res. Commun. 106, 270–275.
[25] Zeiger, R.S., Yurdin, D.L. and Colten, H.R. (1976) J. Allergy Clin. Immunol. 58, 172–179